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Multiple mechanisms control brain aromatase activity at the genomic and non-genomic level☆

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Abstract

Evidence has recently accumulated indicating that aromatase activity in the preoptic area is modulated in parallel by both slow (hours to days) genomic and rapid (minutes to hours) non-genomic mechanisms. We review here these two types of control mechanisms and their potential contribution to various aspects of brain physiology in quail. High levels of aromatase mRNA, protein and activity (AA) are present in the preoptic area of this species where the transcription of aromatase is controlled mainly by steroids. Estrogens acting in synergy with androgens play a key role in this control and both androgen and estrogen receptors (ER; α and β subtypes) are present in the preoptic area even if they are not necessarily co-localized in the same cells as aromatase. Steroids have more pronounced effects on aromatase transcription in males than in females and this sex difference could be caused, in part, by a sexually differentiated expression of the steroid receptor coactivator 1 in this area. The changes in aromatase concentration presumably control seasonal variations as well as sex differences in brain estrogen production. Aromatase activity in hypothalamic homogenates is also rapidly (within minutes) down-regulated by exposure to conditions that enhance protein phosphorylation such as the presence of high concentrations of calcium, magnesium and ATP. Similarly, pharmacological manipulations such as treatment with thapsigargin or stimulation of various neurotransmitter receptors (α-amino-3-hydroxy-methyl-4-isoxazole propionic acid (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA)) leading to enhanced intracellular calcium concentrations depress within minutes the aromatase activity measured in quail preoptic explants. The effects of receptor stimulation are presumably direct: electrophysiological data confirm the presence of these receptors in the membrane of aromatase-expressing cells. Inhibitors of protein kinases interfere with these processes and Western blotting experiments on brain aromatase purified by immunoprecipitation confirm that the phosphorylations regulating aromatase activity directly affect the enzyme rather than another regulatory protein. Accordingly, several phosphorylation consensus sites are present on the deduced amino acid sequence of the recently cloned quail aromatase. Fast changes in the local availability of estrogens in the brain can thus be caused by aromatase phosphorylation so that estrogen could rapidly regulate neuronal physiology and behavior. The rapid as well as slower processes of local estrogen production in the brain thus match well with the genomic and non-genomic actions of steroids in the brain. These two processes potentially provide sufficient temporal variation in the bio-availability of estrogens to support the entire range of established effects for this steroid.

Keywords: Estrogen synthase; Preoptic area; Sexual behavior; Protein phosphorylations; Calcium; Glutamate receptors

1. Introduction

Many biological effects of androgens in the brain require the local conversion of these steroids to an estrogen [11,63,75,77,101]. Although this phenomenon was discovered over 25 years ago [75,78] and is axiomatic among neuroendocrinologists, the implications and significance of this fact is still not widely appreciated by many neurobiologists. The conversion is mediated by the enzyme estrogen synthase (aromatase: EC 1.14.14.1), the protein product of gene *Cyp19*. Two of the most common androgens available in the vertebrate brain, testosterone (T) and androstenedione, can in this way be metabolized, respectively, to either 17β-estradiol (E2) or estrone. Several aspects of male reproductive function known to be regulated by gonadal androgens, such as male-typical sexual behaviors and the negative feedback effects of T on luteinizing hormone release, clearly involve the conversion of T to E2 and the subsequent binding of the newly formed estrogen to an estrogen receptor (ER) [11,32,63]. The importance of this process is quite general in that it has been observed in all the vertebrate classes,

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though not surprisingly, species-differences in the level of enzymatic activity have been described [11,29–31]. Because this metabolic conversion step underlies so many actions of T in the male brain, characterizing the distribution and regulation of aromatase in the brain is essential in order to understand the neuroendocrine mechanisms mediating several organismal level phenomena related to male reproduction.

The distribution of aromatase in the brain is reasonably well described based on standard chemical neuroanatomical techniques including immunohistochemistry for the protein (e.g. [22,42,54,93,94]) and in situ hybridization for the mRNA (e.g. [8,61,83,87,92,100,110]) as well as assays of aromatase activity (AA) that have been performed on microdissected brain tissue (e.g. [89,98,102,108]). Avian species, such as the Japanese quail (*Coturnix japonica*), have been especially helpful due to the fact that the concentration of the enzyme is relatively high thus facilitating detection [95,97,98]. A general pattern of distribution has now been described in the brain with several features common to all vertebrates as, for example, especially high concentrations in the preoptic area, the ventro-medial hypothalamus and the bed nucleus striae terminalis (BST) [76].

Studies of the regulation of the enzyme activity in the brain have originally focussed on changes in the enzyme concentration. It was discovered early on that AA can itself be regulated by gonadal steroids [88,89,102]. A substantial body of evidence now exists indicating that increased gene transcription resulting from androgens or estrogens binding to their cognate receptors is one important way in which AA is regulated and this regulation in turn determines the local concentration of estrogens available in various brain areas [1,20,85,86,90]. This regulatory mechanism is relatively slow, at least as compared with other chemical messenger systems in the brain, such as neuropeptides or neurotransmitters. Because of this, it has been implicitly assumed that variation in estrogen availability is usually correlated with changes in phenotypic state that are characteristic of sex differences, seasonal variation or developmental variation.

The activity of many enzymes both in the brain and in other tissues is also frequently regulated much more rapidly via processes such as protein phosphorylation [4,40]. We recently obtained evidence indicating that AA in preoptic-hypothalamic homogenates of quail brain is rapidly down-regulated by exposure to conditions that enhance Ca^{2+}-dependent protein phosphorylation (addition of Ca^{2+}, Mg^{2+}, ATP) [13,14]. AA was also down-regulated in quail preoptic-hypothalamic explants exposed to high Ca^{2+} levels following a K^{+}-induced depolarization or the stimulation of glutamate receptors. This down-regulation occurred rapidly (within minutes) and was fully reversible [14].

Pharmacological studies of the effects of several protein kinase activators and inhibitors and of protein phosphatase inhibitors on the down-regulation of AA produced by the addition of Ca^{2+}, Mg^{2+} and ATP confirm the implication of phosphorylation processes in these rapid changes in aromatase activity [13]. More recently, additional experiments were carried out to assess whether the aromatase molecule itself is phosphorylated, rather than an eventual intermediary regulatory protein. Aromatase from quail preoptic-hypothalamic homogenates was purified by immunoprecipitation and identified by Western blotting. The presence of phospho-Serine (Ser-P), phospho-Threonine (Thr-P) and phospho-Tyrosine (Tyr-P) groups on the purified aromatase was confirmed by specific Western blotting analysis. These data thus confirm that several kinases can work rapidly to control AA and hence estrogen production, within minutes, in a Ca^{2+}-dependent manner, in the preoptic-hypothalamic region. Putative sites of phosphorylation can accordingly be detected on the deduced amino acid sequence of the quail aromatase.

These observations of a rapid regulation of AA are especially interesting given that in recent years rapid effects of estrogen in the brain have been reported indicating that estrogen itself can either act in a relatively slow way by binding to intracellular receptors and modulating transcription or in a relatively rapid manner via effects on cell membranes. With these new data, a scenario is emerging in which estrogen in the brain can both act and be regulated in a relatively slow manner (as has been known for many years) and in a relatively fast manner [17,18].

In this section, we shall review these two quite different modes of regulation of aromatase activity that result from changes in the concentration of the enzyme or from changes in its conformation and phosphorylation status. As in our previous work, we used preoptic-hypothalamic homogenates derived from Japanese quail, a species which has many advantages as a model system for the investigation of brain AA in relation to male reproduction such as the easy visualization of the enzyme via immunohistochemistry [22,44] and the detailed information we have about the functional significance of brain AA [16]. The mechanisms underlying these controls will be considered as well as their potential significance for the control of many physiological and behavioral processes at the organismal level.

2. Steroid-dependent transcriptional mechanisms control aromatase concentration in the brain

Studies in another avian species, the ring dove (*Streptopelia risoria*) were among the first to demonstrate that in most parts of the brain, including the preoptic area and hypothalamus, sex steroids have a prominent effect on aromatase activity [1,20,52,53,102]. This notion has now been extended to a wide variety of vertebrate species. T increases AA in the preoptic area of all species of higher vertebrates examined so far. This effect appears to be largely mediated by the interaction of the steroid with androgen receptors (AR) in rats [88–90] while in birds it is the result of an action of locally produced estrogens [53]. A component of the regulatory mechanism, based on studies of quail, involves a synergism between non-aromatizable androgens

and estrogens and this synergism can be observed in the three different measures with which aromatase has been studied (the mRNA, the protein and the enzyme activity) [1,27,47,96]. Similar results have been described in another avian species, the ring dove [53] and as well as in mammals [85,89,90]. Together these studies provide a consistent pattern of results indicating that both androgens and estrogens participate to the control of aromatase in birds and mammals but that androgens play the major role in mammals while estrogens play the major role in birds. Studies in the quail preoptic area, in particular, have demonstrated an excellent match between T-induced increases in aromatase mRNA, aromatase protein and aromatase activity suggesting that steroid-induced changes in AA result largely from changes in the transcription of the enzymatic protein [13,20].

In all avian species investigated so far, and in quail, in particular, aromatase control is mainly effected by estrogens and androgens only play a subsidiary role. The most parsimonious cellular model that could explain this control of aromatase synthesis assumes that when entering its target cells, T is aromatized in the cytoplasmic compartment into an estrogen which would then bind to estrogen receptors while non-metabolized T would bind to androgen receptors. These two types of occupied receptors would then activate the transcription of the aromatase gene. Double label immunocytochemistry has demonstrated that a large number of aromatase-immunoreactive (ARO-ir) cells in the quail preoptic area co-express immunoreactive AR [24]. Surprisingly, however, the estrogen-synthesizing enzyme is not systematically co-localized with ER of the α subtype (ERα), although both the enzyme and the receptors are expressed in the same brain areas (e.g. the preoptic area, the bed nucleus striae terminalis, the ventro-medial nucleus of the hypothalamus) [25]. For example, most ARO-ir neurons in the ventro-medial hypothalamus contain immunoreactive ERα, but the percentage of this co-localization is far lower in the medial part of the preoptic area (approximately 18%) and bed nucleus striae terminalis (approximately 4%). Similar co-localization results have been reported in rodents [106,107].

A second type of estrogen receptor, called ERβ has now been identified in mammals [58,59] and has also been cloned in quail [43]. The analysis of its neuroanatomical distribution by in situ hybridization with antisense oligonucleotide probes demonstrated an extensive distribution of the ERβ mRNA throughout the rostral-caudal extent of the hypothalamus, in the mesencephalic nucleus intercollicularis and in the telencephalic nucleus taeniae [43]. An intense hybridization signal outlined, in particular, the medial part of the preoptic area, the rostral part of the nucleus striae terminalis (BST) as defined by Aste et al. [7] and the tuberal hypothalamus, i.e. the three brain areas that contain the densest populations of ARO-ir neurons [22,44] (see Fig. 1). It is therefore conceivable that ERβ is co-localized with aromatase in a large number of neurons and that estrogens control aromatase synthesis in an intracrine manner after binding to ERβ. However, the co-existence of ERβ and aromatase in the same brain area does not necessarily prove that these two antigens are co-localized in the same cells, as already observed for ERα and additional work is now required to investigate the extent of this co-localization, if any. However, even if these studies fail to demonstrate the presence of ER in ARO-ir cells, estrogens produced in the brain could still affect aromatase activity by diffusing to adjacent cells that contain ER.

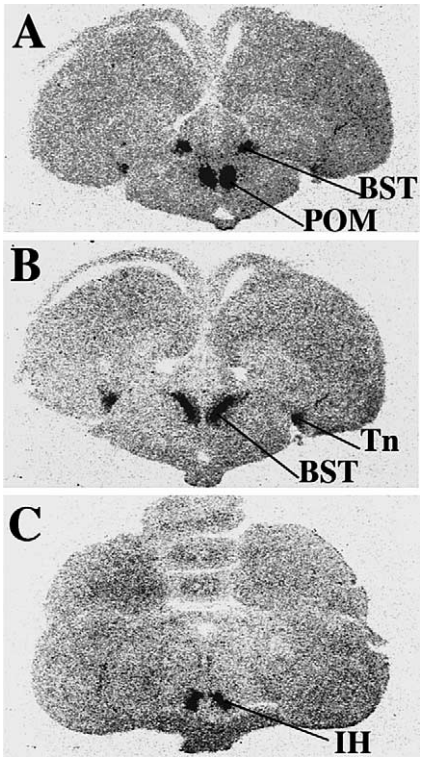

Fig. 1. Autoradiograms of coronal sections in the quail brain illustrating the distribution of the ERβ mRNA visualized by in situ hybridization with the combination of probes labeled with ^{33}P (see [43] for detail). Sections A through C are presented in a rostral to caudal order. A very dense expression is observed in nuclei that contain high densities of aromatase-immunoreactive cells such as the medial preoptic nucleus (POM), bed nucleus striae terminalis (BST) and infundibular hypothalamus (IH). ERβ mRNA is also present in high density in the nucleus taeniae (Tn) that contains only a small population of aromatase-positive cells.

Alternatively, estrogens could also modify AA and transcription by affecting inputs to aromatase cells in a manner reminiscent of what has been described in much more detail for the regulation of GnRH neurons (e.g. [28,41,49,112]; see [1] for a detailed discussion of this idea).

3. Sex differences in aromatase concentration and in expression of the steroid receptor coactivator 1

Male and female quail do not respond in the same way to a similar treatment with T. In particular, female quail never

show male-typical copulatory behavior even after ovariectomy and exposure to doses of exogenous T that are fully effective in activating this behavior in males [2,26]. In quail like in many other avian and mammalian species, the aromatization of androgens into estrogens plays a key role in the control of many physiological processes related to reproduction and in particular, in the activation by T of male sexual behavior [3,16,81]. Interestingly, the activity of aromatase is sexually differentiated in quail (see [3,81] for review) as it is in rats [84,91] and this enzymatic difference could thus play an important role in the control of the behavioral sex dimorphism.

Aromatase activity is significantly higher in the preoptic area of adult sexually mature male quail than in females of the same age and gonadectomy reduces this enzyme activity to basal levels in both sexes. However, a same replacement therapy with T differentially restores the enzymatic levels in males and females so that gonadectomized T-treated males recover a significantly higher AA than females despite the fact that birds of both sexes were exposed to a similar endocrine milieu [97]. The functional consequences of this differential induction of aromatase by T are relatively simple to envision (differential activation of copulatory behavior) but its mechanistic control remains unclear at present. Biological effects of steroids result largely from specific interactions with intracellular receptors located in the target tissues [67,68]. Steroid hormones receptors are members of the nuclear receptors superfamily of ligand-inducible transcription factors that include receptors for estrogens, androgens, progestins, glucocorticoids, thyroid hormones, Vitamin D3, retinoic acids and 9-*cis* retinoic acids along with numerous orphan receptors. When exposed to their ligand, the steroid receptors dimerize, bind to hormone response elements located on the DNA and regulate the gene transcription function of receptors [55,111]. Recent studies reveal that receptors undergo a characteristic conformational change upon hormone activation that allows the recruitment of several proteins implicated in the transcriptional activation (review in [62,69]). The function of these proteins is to increase (co-activators) or decrease (co-repressors) the binding to the response elements and thus the transcriptional activity of the receptors. The steroid receptor coactivator (SRC) family consists of three newly identified co-activators designed SRC-1 [80], SRC-2/TIF2/GRIP1 [51,109] and SRC-3/p/CIP/RAC3/ACTR/TRAM1/AIB1 [5,64,104,113].

SRC-1, in particular, seems to be implicated in the control of the transcriptional activity of sex steroids. For example, the organizing effects of estrogens on the brain and on sexual behavior are markedly affected by SRC-1 antisense oligonucleotide injection [9] and a similar antisense treatment into the ventro-medial nucleus of the hypothalamus interferes with the expression of progesterone-facilitated lordosis behavior in female rats [73]. We therefore hypothesized that the differential transcription of aromatase in males and females in response to a same treatment with sex steroid could be attributed to a differential expression of SRC-1. Until recently, no antibody was available to analyze the distribution of SRC-1 in the avian brain and the sequence of this protein had not been identified in any avian species. We therefore decided to clone SRC-1 by reverse transcriptase-chain polymerase reaction (RT-PCR) amplification on mRNA from the quail brain and could in this way identify a cDNA that is largely homologous with the SRC-1 sequence previously described in mammals.

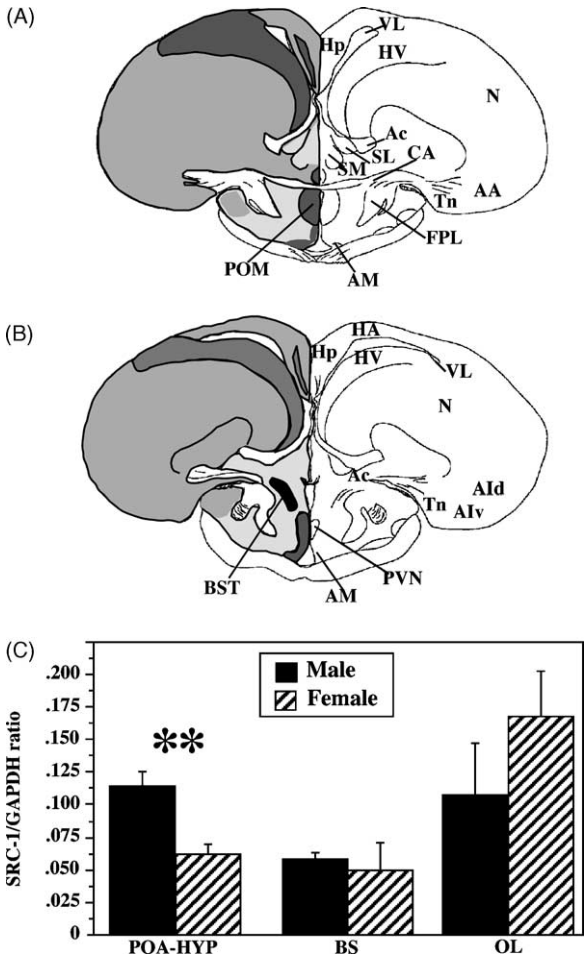

Fig. 2. (A and B) Schematic drawings of coronal sections through the quail brain at the level of the medial preoptic nucleus (A) or of the bed nucleus striae terminalis (B) illustrating the relative intensity of SRC-1 labeling by in situ hybridization. Five gray levels (white to dark gray) are used to describe the relative intensity of the SRC-1 expression in different brain areas. (C) Bar graphs illustrating the SRC-1/GAPDH mRNA ratios in the preoptic area (POA)-hypothalamus, brain stem (BS) and optic lobes (OL) of male and female quail as obtained by real time quantitative PCR of cDNA samples. $^{**}P < 0.005$ by comparison with the same region in males (see [34] for detail). *Abbreviations*: AA, archistriatum anterior; Ac, nucleus accumbens; AId, archistriatum intermedium pars dorsalis; AIv, archistriatum intermedium pars ventralis; AM, nucleus anterior medialis hypothalami; BST, nucleus striae terminalis; CA, anterior commissure; FPL, fasciculus prosencephali lateralis; HA, hyperstriatum accessorium; Hp, hippocampus; HV, hyperstriatum ventrale; N, neostriatum; POM, nucleus preopticus medialis; PVN, nucleus paraventricularis; SL, nucleus septalis lateralis; SM, nucleus septalis medialis; Tn, nucleus taeniae; VL, ventriculus lateralis.

Total RNA was isolated from Japanese quail brains and reverse-transcribed. We then amplified by RT-PCR a 3411 bp fragment extending from the PAS-B region to the Activating Domain-2 of the protein [34]. The quail SRC-1 was shown to be closely related to the mammalian (m) SRC-1 and to the SRC-1 sequence that was identified in a related avian species, the domestic chicken while this work was in progress [6]. The quail SRC-1 contains a high proportion of GC nucleotides (62.5%). Its amino acid sequence presents 70% identity with mammalian SRC-1 and contains the three conserved LXXLL boxes involved in the interaction with nuclear receptors.

RT-PCR demonstrated in both males and females a similar high level of expression in a variety of brain areas throughout the brain. In addition, in situ hybridization utilizing a mixture of four digoxigenin-labeled oligonucleotides confirmed at the cellular level this widespread but heterogeneous distribution of SRC-1 mRNA in the brain [34]. These studies also identified a particularly dense SRC-1 expression in several steroid-sensitive areas that play a key role in the control of male sexual behavior. These brain areas include the two main nuclei that contain ARO-ir cells, namely the medial preoptic area and bed nucleus striae terminalis (Fig. 2).

Although we do not yet know whether SRC-1 is co-localized with aromatase in these brain areas, it is of interest to note that a first set of quantitative PCR studies indicated that males express significantly higher levels of SRC-1 in the preoptic area-hypothalamus than females [33]. This differential expression of the co-activator could thus explain at least in part the differential induction by steroids of aromatase expression in the preoptic area. Additional studies are currently in progress to test this hypothesis. However, independent of the outcome of these studies, the very dense expression of SRC-1 in limbic nuclei that are associated with the control of male sexual behavior, as compared to nearby areas that have much lower levels of expression, supports the notion that this coactivator plays a significant role in the activation of this behavior.

4. Phosphorylations rapidly affect aromatase activity

The changes in aromatase activity described so far result largely, if not exclusively, from variations in the transcription and thus concentration of the enzyme. As such, they occur over relatively long periods. It has been shown, for example, that it takes approximately 8 h of exposure to exogenous T to double the enzymatic activity in the preoptic area of castrated male quail [23]. The activity of many enzymes, such as Tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, can be modified more rapidly by conformational changes in the enzyme molecule such as phosphorylations that are catalyzed by specific kinases in the presence of suitable concentrations of ATP and the divalent cations, Ca^{2+} and Mg^{2+} [4,40]. Because previous studies had implicated divalent cations in the control of AA [50,79,103], we investigated whether increases in ATP, Ca^{2+} and Mg^{2+} concentrations could affect AA in male quail brain homogenates.

A profound inhibition of AA was observed in quail brain homogenates that had been preincubated with increased but physiological concentrations of ATP, $Mg^{2+}$and Ca^{2+}. This inhibition was, however, blocked by agents that chelate divalent ions such as EGTA or EDTA [14].

Additional support to the idea that this enzymatic inhibition is actually mediated by phosphorylation processes was obtained based on pharmacological experiments utilizing inhibitors of Serine/Threonine or Tyrosine phosphorylations. The decrease in AA observed in the presence of increased concentrations of ATP, Ca^{2+} and Mg^{2+} concentrations was indeed almost completely blocked in the presence of genistein (inhibitor of Tyrosine kinases) or of staurosporine (inhibitor of Serine/Threonine kinases) [13]. Subsequent experiments utilizing a variety of more specific kinase inhibitors confirmed that changes in AA result from phosphorylation processes and indicated that these phosphorylations are catalyzed mainly by protein kinase C (PKC), protein kinase A (PKA) and to a lower extent by one type of calmodulin kinase (CamK) (see [13,15]).

In addition, rapid (within minutes) and reversible inhibitions of AA were also observed in hypothalamic explants incubated in vitro and exposed to conditions that lead to an increase in the intracellular Ca^{2+} levels such as a K^{+}-induced depolarization, a treatment by thapsigargin or the exposure to glutamate agonists (kainate, α-amino-3-hydroxy-methyl-4-isoxazole propionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA)) [13,14]. These data therefore clearly support the notion that the local production of estrogens in the brain can be rapidly changed by Ca^{2+}-dependent phosphorylations based on variations in neurotransmitter activity. A variety of environmental stimuli could thus potentially affect the bio-availability of estrogens in the brain with a high degree of anatomical specificity.

5. Phosphorylations directly concern the aromatase protein

Because all experiments described above were carried out on crude brain homogenates, they did not determine whether the phosphorylations controlling AA directly affect the aromatase enzyme itself or another co-existing protein that would secondarily regulate aromatase. To address this question, aromatase from quail POA homogenates was purified by immunoprecipitation and probed by Western blotting techniques for the presence of phosphorylated residues on the aromatase protein band [15].

During these experiments, aromatase was immunoprecipitated with an anti-aromatase antibody [44] in aliquots of homogenates that had been prepared in control buffer and then preincubated for 15 min at 37 °C either in the same control conditions or in the presence of 1 mM EGTA or of ATP/Mg/Ca, i.e. in conditions corresponding to the minimal

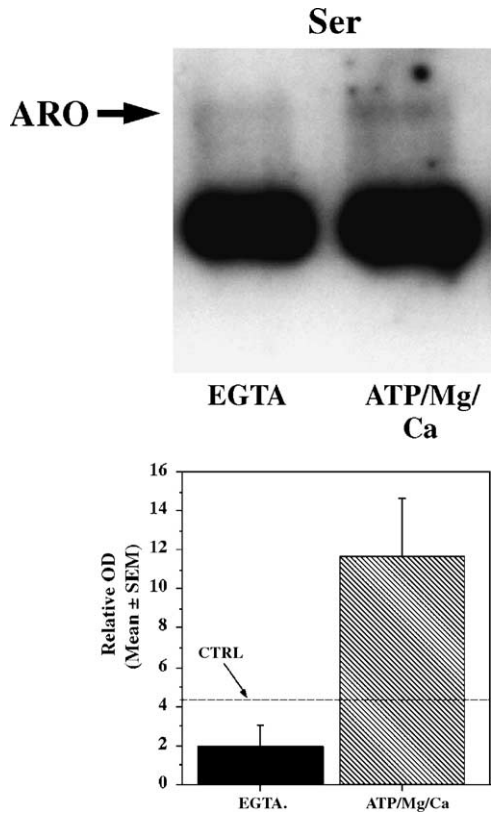

Fig. 3. Detection by Western blot of Serine phosphate (Ser), at the level of the aromatase (ARO) band purified by immunoprecipitation followed by electrophoresis. After pre-incubation of POA-hypothalamus homogenates in 2 different experimental conditions (1 mM EGTA [EGTA] or 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM ATP [ATP/Mg/Ca]), aromatase was purified by immunoprecipitation and submitted to Western blot analysis with antibodies directed against Ser-P. The bottom panel presents the mean relative optical densities of the bands corresponding to the phosphorylated amino acid in the two experimental conditions (means of eight experiments). The mean level observed in control conditions (CTRL) is indicated by the dotted line (prepared from data in [15]).

and maximal phosphorylation levels, respectively. Blots that had been prepared in parallel were then probed with antibodies directed against either aromatase, Ser-P, Thr-P or Tyr-P (Fig. 3).

In all experiments, phosphorylated residues (Ser-P, Thr-P and Tyr-P) were identified by the specific antibodies at the level of the electrophoresis band corresponding to aromatase (i.e. at a molecular weight of approximately 60 kDa). The blots also suggested that the intensity of these signals was denser when homogenates that had been preincubated with ATP/Mg/Ca than when they had been incubated in EGTA (Fig. 3). These differences were quantified by image analysis measuring the optical density (gray levels from 0 to 256) of the bands corresponding to the phosphorylated amino acids and of a similar area located on the same gel and in the same lane but at a lower level (background). The difference between these two measures (relative optical density, ROD), was then compared in the two experimental conditions by a paired Student's *t*-test since aliquots exposed to EGTA or ATP/Mg/Ca came from the same homogenate and were analyzed on the same gel. The average ROD was almost invariably higher in aliquots that had been exposed to phosphorylating conditions (pre-incubation with ATP/Mg/Ca) than in aliquots exposed to the calcium chelating agent (EGTA). In the case of phospho-Serine that was studied in eight independent experiments, the ROD difference between these two conditions was statistically significant. These experiments therefore indicate that phosphorylation processes that are enhanced in the presence of ATP/Mg/Ca directly affect the aromatase protein.

6. Consensus phosphorylation sites on the aromatase molecule

This conclusion was also supported by the fact that multiple phosphorylation consensus sites are present in the sequences of aromatase that had been previously cloned in mammals (e.g. [35,46,71]) and in birds [70,99]. Quail aromatase had, however, been partly sequenced only [48] so that it was impossible to determine whether all these consensus sites were conserved in this species.

To address this question, we recently cloned the quail aromatase cDNA by a combination of 5′- and 3′-RACE utilizing primers that were derived from the two ends of the previously cloned sequence [15]. We identified in this way two nucleotidic sequences partly overlapping with the previously cloned portion of the quail aromatase molecule. The translation start codon was assumed to be the same as the codon used in other species. The open reading frame of the quail aromatase identified by assembling these partial sequences covers a total of 1541 bp and encodes a 490 amino acid protein which corresponds to the entire aromatase molecule as identified in other species of higher vertebrates with the exception of the last 6 amino acids at the 3′ end. This sequence contains all previously described functional domains that have been identified in the mammalian and avian aromatase [99], namely, the putative transmembrane domain, I-helix, steroid-binding domain, aromatic region and heme-binding domain (see Fig. 4).

Alignment with aromatase sequences previously identified in avian (e.g. chicken [65]) or mammalian (e.g. mouse [105]) species revealed a high degree of homology (e.g. 95% with chicken or 70% with mouse; see Fig. 4), especially in the two putative steroid-binding domains where homology between quail and the other avian species cloned to this date (chicken and zebra finch [99,105]) is 100% and there is only one amino acid difference with the human or mouse sequences [46,105].

A total of 15 significant consensus phosphorylation sites were identified on the quail aromatase with the NetPhos 2.0 software (http://www.cbs.dtu.dk/services/NetPhos/) they concern 8 of the 24 Serine, 5 out of the 20 Threonine and 2 out of the 14 Tyrosine residues (see [15]). Confronting these consensus sequences with consensus sequences that

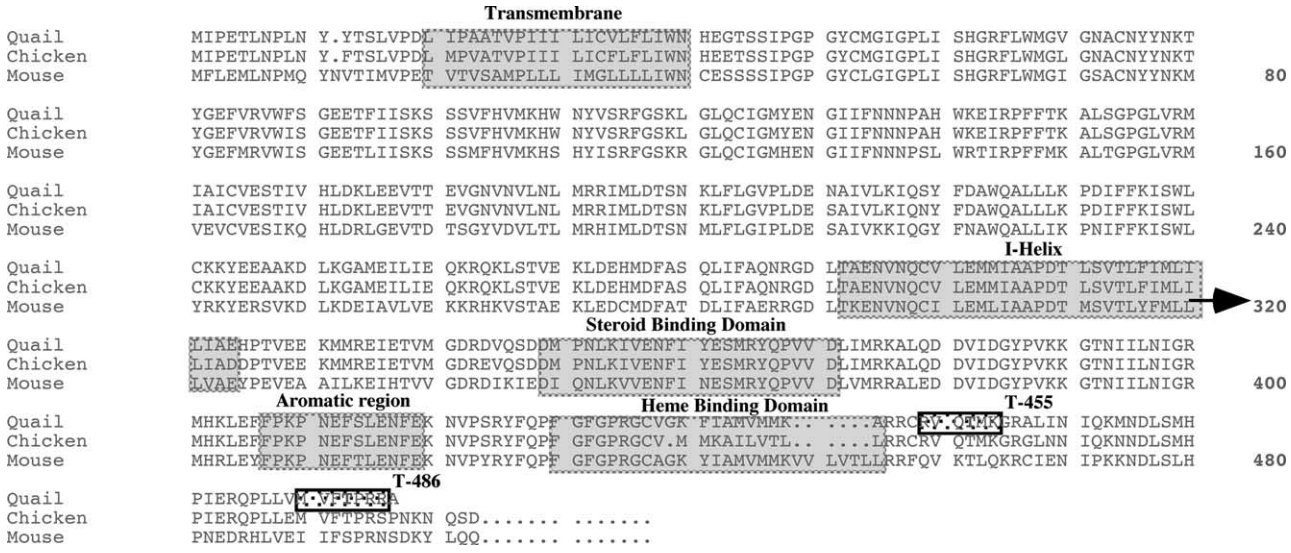

Fig. 4. Comparison of the amino acid sequences of aromatase in quail [15,48], chicken [65] and mouse [105]. Functional domains as described by Shen et al. [99] are enclosed in gray boxes. The phosphorylation consensus sites that displayed a consensus score higher than 0.5 (minimal significance level) and were at the same time considered as consensus sequences corresponding to the protein kinases that affect aromatase activity during the pharmacological experiments (i.e. PKA and PKC, see text) are also indicated in black boxes (Threonine (T) 455 and 486). Residues are numbered on the right side based on the mammalian sequence that does not take into account the deletions observed in the quail and chicken aromatase. The two Threonine residues in the quail sequence that are potential phosphorylation sites are additionally numbered based on the quail sequence (see [15] for detail).

are specific for the particular protein kinases that were shown during pharmacological experiments to affect aromatase activity (i.e. PKC and PKA) indicated, however, that only two of these 15 residues have at the same time a consensus phosphorylation score higher than the critical value of 0.5 and a sequence that fits the PKA and/or PKC specificity. These sites correspond to the Threonine residues located at positions 455 and 486 of the quail aromatase sequence (corresponding to positions 462 and 493 in human or mouse aromatase due to deletion in quail in positions 12 and 449–454, see Fig. 4). These two positions also correspond to the two residues that display some of the highest consensus scores (0.977 and 0.987, respectively) and these parts of the sequence are well conserved across species. It is therefore tentatively suggested that the phosphorylations of the aromatase protein that affect its enzymatic activity are likely to affect one or both of these Threonine residues. The fact that they are located in the vicinity of the active site of the enzymatic molecule further supports this conclusion.

7. Transmitters directly affect aromatase-expressing cells to modulate enzyme activity

Anatomical studies first suggested that catecholamines (dopamine and noradrenaline) control AA due to the frequent apposition of Tyrosine hydroxylase-immunoreactive fibers and punctate structures on aromatase-immunoreactive cells in the quail medial preoptic nucleus (POM) and bed nucleus striae terminalis [21]. Pharmacological work confirmed this idea. AA in quail brain homogenates is inhibited in the presence of dopamine and of various dopaminergic agonists or antagonists [10] and these compounds similarly inhibit AA expressed in preoptic quail explants maintained in vitro [1,12]. These dopaminergic effects are, however, somewhat peculiar in the sense that they are observed in the presence of agonists as well as antagonists of the D1 or D2 receptors so that they are probably not mediated by a direct interaction with classical dopaminergic receptors [1,12]. The underlying mechanism(s) mediating these effects thus remain(s) to be identified (interaction with specific receptors located on the aromatase neurons, on adjacent cells, direct interaction with the enzyme activity, ... see [12] for a detailed discussion) but these data clearly indicate that catecholamines can markedly affect estrogen synthesis in the brain.

Other transmitters, in particular glutamate, have similar effects. The addition of ionotropic excitatory amino acid receptor agonists, such as kainate, α-amino-3-hydroxymethyl-4-isoxazole propionic acid or *N*-methyl-D-aspartate markedly depresses AA in hypothalamic explants, presumably via the calcium influx that they stimulate [14]. We previously demonstrated by immunocytochemical techniques that these three types of glutamate ionotropic receptors are present in the aromatase rich areas of the quail brain including POM and BST [37] but the specific anatomical relationships between aromatase and these receptors are still largely unexplored and in particular, no study ever assessed whether these receptors are present at the surface of aromatase-expressing cells.

Due to difficulties in addressing this question by double label immunocytochemistry (antibody against neurotrans-

mittters are not available or poorly cross-react with avian antigens), we decided to investigate this question by electrophysiological techniques in quail brain slices by coupling intracellular recordings with injections of biocytin that could later be used to identify the cells that were recorded and analyze their phenotype by immunocytochemistry [36].

During these experiments, coronal 300 μM-thick slices containing the POM were prepared with a vibratome, collected in cold artificial cerebrospinal fluid (ACSF) and transferred into a recording chamber. Intracellular recordings in the bridge balance mode were obtained with microelectodes filled with K-acetate and biocytin. At the end of the electrophysiological recordings, slices were fixed with paraformaldehyde, cryoprotected in sucrose 20% and later stained by immunocytochemistry for aromatase with a rabbit antibody raised against quail recombinant aromatase

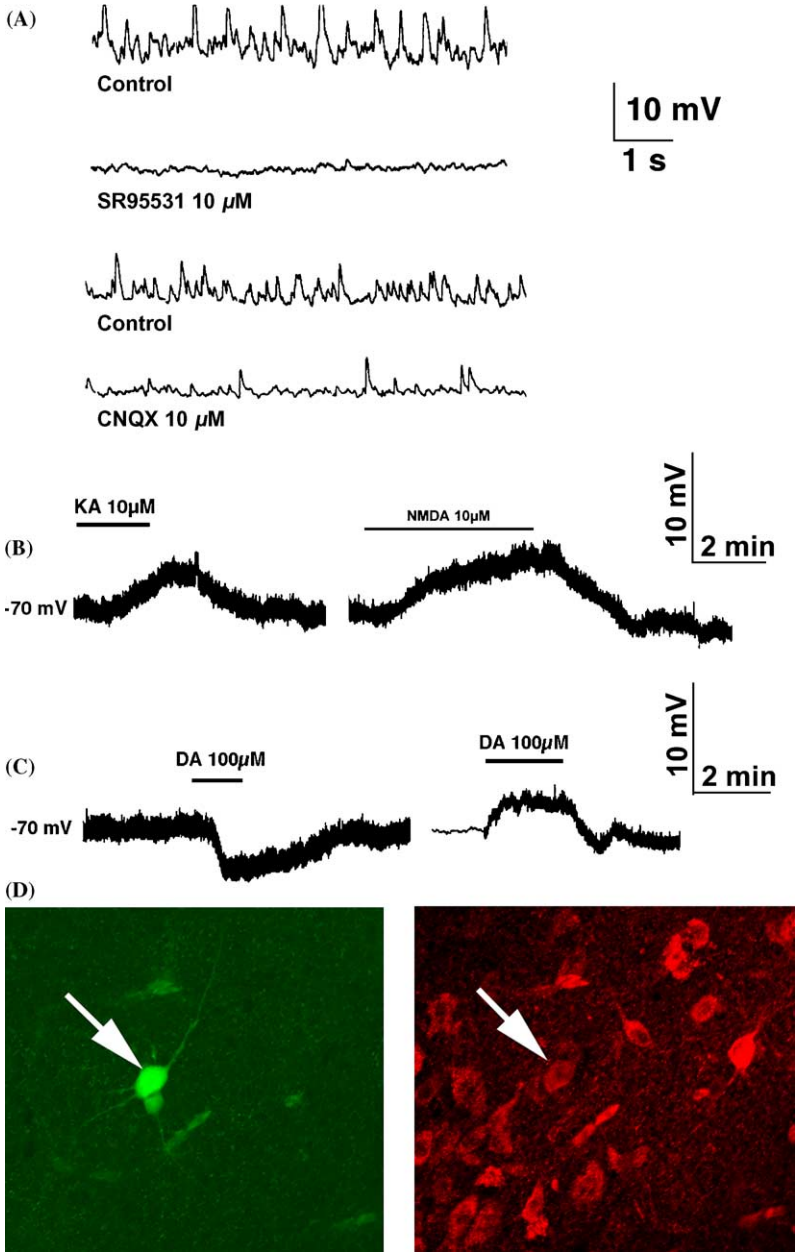

Fig. 5. (A) Effects of a $GABA_A$ or an AMPA/kainate receptor antagonist (SR95531 and CNQX, respectively) on spontaneous post-synaptic potentials observed in control conditions in neurons of the quail medial preoptic area. Recordings were obtained at a membrane potential of −70 mV clamped by injection of a continuous current of ±100 pA. (B) Effect of two glutamate agonists, kainate (KA) and NMDA on the membrane potential of neurons in the quail medial preoptic area. In all recorded cells, both kainate and NMDA produced a depolarization of 8–10 mV. Recordings shown here were obtained at a membrane potential of −70 mV clamped by injection of a continuous current of ±100 pA and under continuous superfusion of 0.5 μM TTX. (C) The superfusion of dopamine (DA 100 μM) produced either a hyperpolarization or a depolarization of neurons in the quail medial preoptic area. (D) Illustration of a cell (arrow) injected with biocytin during electrophysiological recordings (left) that was proved to be aromatase-immunoreactive by immunocytochemical staining at the end of the electrophysiological experiments (right).

(kindly provided by N. Harada, Fujita Health University, Toyoake, Japan; see [44]). Stained slices were observed under confocal laser microscopy that allowed discrimination of aromatase-containing cells stained in red by rhodamine and biocytin-injected cells stained in green by FITC.

The vast majority of POM neurons displayed a spontaneous synaptic activity due to the activation of $GABA_A$ and AMPA receptors. This activity could be suppressed by the addition of a mixture of glutamate and GABA antagonists (Fig. 5A).

Intracellular recordings were then obtained in the presence of 0.5–1 μM tetrodotoxin (TTX) resulting in a complete suppression of synaptic activity so that any changes in membrane potential could be attributed to a direct effect on the cell under investigation. In all cells ($n = 8$–11), clear variations of membrane potential were obtained when slices were superfused with 10 μM kainate (mean depolarization: 10.4 ± 1.0 mV) or 10 μM NMDA (mean depolarization: 8.7 ± 1.4 mV; Fig. 5B). The $GABA_A$ receptors agonist, isoguvacine (100 μM) also affected the membrane potential clamped at −70 mV by injection of a continuous current of approximately 100 pA in the majority of neurons [36].

Similarly dopamine affected most recorded cells but hyper- as well as de-polarizations were observed in this case due presumably to the opposite effects of the different subtypes of receptors on cell physiology (Fig. 5C) [38]. Interestingly, most effects of dopamine could not be blocked by D1-like nor by D2-like receptor antagonists [38] but were completely suppressed by noradrenergic antagonists. A similar cross-reactivity was also detected during extra-cellular recording experiments: the decreases or increases in firing rates of neurons that were induced by a superfusion with dopamine were not blocked by D1 nor D2 antagonists but were inhibited by α_2- or α_1-antagonists, respectively. These and other experiments indicate that a substantial part of the electrophysiological effects of dopamine in the quail preoptic area are mediated by interactions with noradrenergic receptors [38]. The functional significance of these observations and in particular their implication in the control of aromatase activity remains, however, to be identified.

Taken together, these data indicate that glutamate, GABA and dopamine (or noradrenergic) receptors are expressed on the membrane of neurons in the quail preoptic area and in a large number of cases, multiple receptors are actually present on the membranes of the same cells (see [36]).

Three of the cells recorded in these protocols were found so far to be immunoreactive for aromatase. One example of these cells is illustrated in Fig. 5D. Many of these cells were found to express at the same time receptors sensitive to kainate (3/3), NMDA (2/3) and isoguvacine ($GABA_A$ receptors; 3/3). The presence of these receptors expressed at the surface of aromatase-immunoreactive neurons indicates that aromatase activity could be controlled by a direct action of glutamate or GABA on the aromatase-expressing neurons. Additional studies should however be performed to determine whether these co-localizations of aromatase and neurotransmitter receptors are present in the majority of aromatase neurons, as suggested by the few cells studied so far, or represent an atypical organization.

8. Physiological significance and perspective for future work

Taken together, the data described above indicate that aromatase activity in the quail brain can be controlled by two distinct types of mechanisms. The enzymatic activity can first be enhanced following exposure to sex steroids (T or its metabolite E2). This process is relatively slow. In quail specifically, it has been shown that the treatment of castrates with T only increases AA after several hours. The enzymatic activity doubles in approximately 8 h but only reaches its maximum after 1 of 2 days [23]. These relatively slow changes in aromatase concentration and therefore estrogen production are in good agreement with the classical modes of estrogen action. Estrogens are generally viewed as slow-acting messengers that bind to nuclear receptors which then act as transcription factors. These effects of E2 are also slow and thus consistent with the mechanisms that regulate the local production of estrogens by aromatase in a slow manner (via changes in enzyme concentration).

However, in addition to these changes related to genomic controls of aromatase transcription, the activity of aromatase can also be rapidly affected by calcium-dependent phosphorylations in relation with the activity of several neurotransmitters including glutamate and also possibly dopamine. The cellular mechanisms mediating these changes in the phosphorylation status of aromatase are still not completely understood but it is now firmly established that they result in changes in enzymatic activity that should lead to rapid variations in the concentration of estrogens in the brain.

There is now a broad and rapidly growing literature describing rapid effects of estrogens presumably mediated by interactions with neuronal membranes in the brain. These include electrophysiological responses that are observed within seconds to minutes after application of the steroid (e.g. [45,56,57,66,68,72,74,82]). Estrogens have also been shown to increase in an anatomically specified manner the phosphorylation level of specific proteins. The stimulation of adenyl cyclase leading to an increased phosphorylation by protein kinase A of cyclic AMP response element binding protein (pCREB) represents one common mode of rapid action for estrogens [60]. Accordingly, a marked increase in the level of pCREB has been shown to occur within 15 min in the medial preoptic area and bed nucleus striae terminalis following systemic injection of estradiol in rats (e.g. [114]).

The physiological significance of these rapid actions of estrogens still remains to be evaluated but the fact that they take place in brain areas that are directly relevant to the control of physiological (activity of GnRH neurons) or behavioral aspects of reproduction clearly suggests that they

should play a significant role in the timing of reproductive events. At the behavioral level, recent studies specifically indicate that, in rats, the estrogens are able to activate aspects of male sexual behavior (anogenital investigations and mounts) within less than thirty minutes [39] which raises the possibility that these effects result, at least in part, from non-genomic actions such as the direct phosphorylation of specific proteins.

Similar data are not available for quail to this date so that it is formally impossible to determine what are the physiological and behavioral consequences of the rapid changes in preoptic aromatase activity that have been observed in this species. Experiments addressing these questions are, however, currently in progress. Preliminary electrophysiological studies have identified changes in the responsiveness to kainate in some preoptic neurons following exposure to estradiol. As previously reported in rats [114], it also appears that the level of phosphorylation of CREB is significantly increased in the preoptic area of quail during the first 30 min following an intraperitoneal injection of E2 [19]. These data therefore suggest that the quail brain also can be rapidly affected by changes in E2 concentration and conversely there is every reason to believe that the rapid changes in aromatase activity that we have described in quail also occur in rodents and other mammalian species. The data reviewed above thus suggest that rapid effects of E2 in the brain may be paralleled by rapid changes in bio-availability of this steroid induced by phosphorylations and dephosphorylations of aromatase. Many questions however, remain open at this time. They concern the specific mechanism(s) underlying these phosphorylations (positive identification of the residues whose phosphorylation controls aromatase activity, of the kinases involved, of the intracellular signaling cascades controlling these kinases) as well as the identification of the transmitters pathways afferent to the aromatase cells or the determination of the full functional significance of these changes in enzymatic activity. The mechanisms described above pose many new questions for investigators in the field of (behavioral) neuroendocrinology.

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